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14. ABSTRACT

To create PEDF based therapy for hormone-refractory CaP we have proposed to generate short synthetic peptides corresponding to the 34-mer anti-angiogenic epitope of PEDF. The 3D structure of the 34-mer peptide was analyzed using Protean software in terms of relative hydrophobicity, charge distribution, and antigenic index. Three synthetic peptides covering the 34-mer fragment were generated and tested for the ability to reproduce anti-angiogenic effect of PEDF. All peptides (14, 18 and 23-mer) inhibited FGF-induced endothelial cell migration. Dose-response curves were generated and the potency of the peptides compared to native PEDF and the 34-mer. Neither of the peptides showed signs of toxicity at the doses tested. Although all peptides showed anti-angiogenic activity in migration assay only 18 and 23-mer induced apoptosis in endothelial cells. 18-mer peptide also blocked neovascularization induced by FGF as demonstrated in corneal assay. This peptide will be further tested in vivo in mouse model for ability to inhibit prostate tumor growth.

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PEDF, anti-angiogenic inhibitor, peptides

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Table of Contents

Introduction..... 4

Body..... 4

Key Research Accomplishments..... 9

Reportable Outcomes..... 9

Conclusions..... 9

References..... 9

Appendices.....10

INTRODUCTION:
Pigment epithelial-derived factor (PEDF) is a potent natural angiogenesis inhibitor. We have recently mapped PEDF anti-angiogenic activity to its 34-mer N terminal peptide (residues 24-57). This peptide retains PEDF anti-angiogenic properties: it reproduces signaling events in endothelial cell (EC), elicits EC apoptosis and blocks migration. Forced expression of 34-mer peptide delays the growth of prostate carcinoma. To create PEDF based therapy for hormone-refractory CaP and other angiogenesis-dependent diseases we have proposed to generate short synthetic peptides corresponding to the 34-mer epitope of PEDF and screen these for the ability to reproduced anti-angiogenic activity of PEDF. In this study we evaluate and compare anti-angiogenic potential of the 34-mer fragment and its internal fragments *in vitro* by their ability to inhibit migration and to induce apoptosis in cultured endothelial cells. We further investigate biologically active peptides for the ability to block neovascularization in vivo. The compound, which retains activity in vivo, will be subjected to the preclinical testing in mouse model of the prostate carcinoma.

BODY:
As indicated in the Statement of Work during this period our task was to evaluate angiosuppresive activity of the shorter peptides generated from anti-angiogenic 34-mer fragment of PEDF. This involved the following steps:
1. I generated series of short peptides to cover the areas of the anti-angiogenic 34-mer with high probability of surface exposure. The 3D structure of the 34-mer peptide was analyzed by Protean software in terms of relative hydrophobicity (i), charge distribution (ii), and antigenic index (iii). The 34-mer C-terminus is strongly hydrophilic, with highly charged central area and high antigenic index, and is likely to interact with a target receptor. Short synthetic peptides covering C-terminus of the 34-mer fragment were designed and generated (see Fig. 1). We have chosen 3 peptides covering the 34-mer fragment of the C-terminus of PEDF peptide: 14-mer (aa 43-57), 18-mer (aa 39-57) and 23-mer (aa 34-57). Synthethic peptides (from Gene Script) were acetylated on the N-terminus and amidated on the C-terminus for stability.

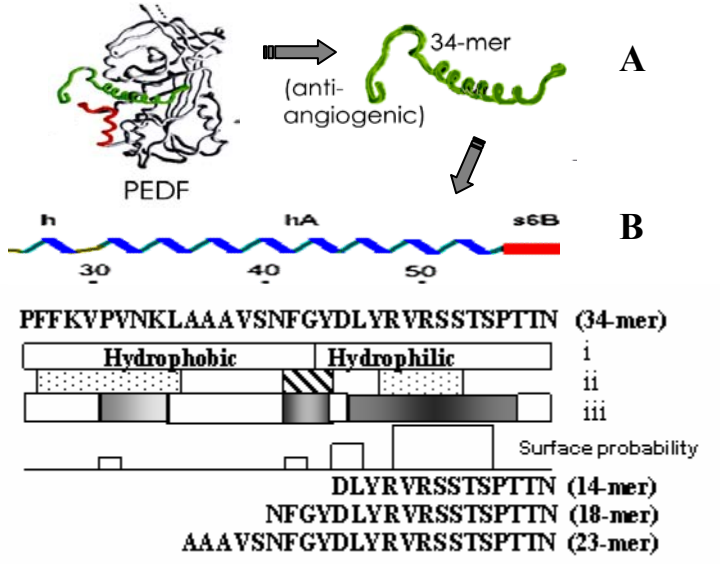


Figure1. Design of PEDF peptides

A. α-carbon stereodiagram of the peptide distribution on the putative interactive interface of PEDF. The 34-mer, marked in green.

B. Linear diagram of the 34-mer fragment. Structural elements and the internal peptides are indicated below the sequence.

2. I determined the effect of synthetic peptides on migration of cultured endothelial cells
All three peptides were thoroughly screened for the ability to inhibit endothelial cells (EC) migration. The results were compared to the recombinant PEDF. We generated dose/response curves for all three peptides (14-mer, 18-mer, and 23-mer) as shown below (Fig. 2).

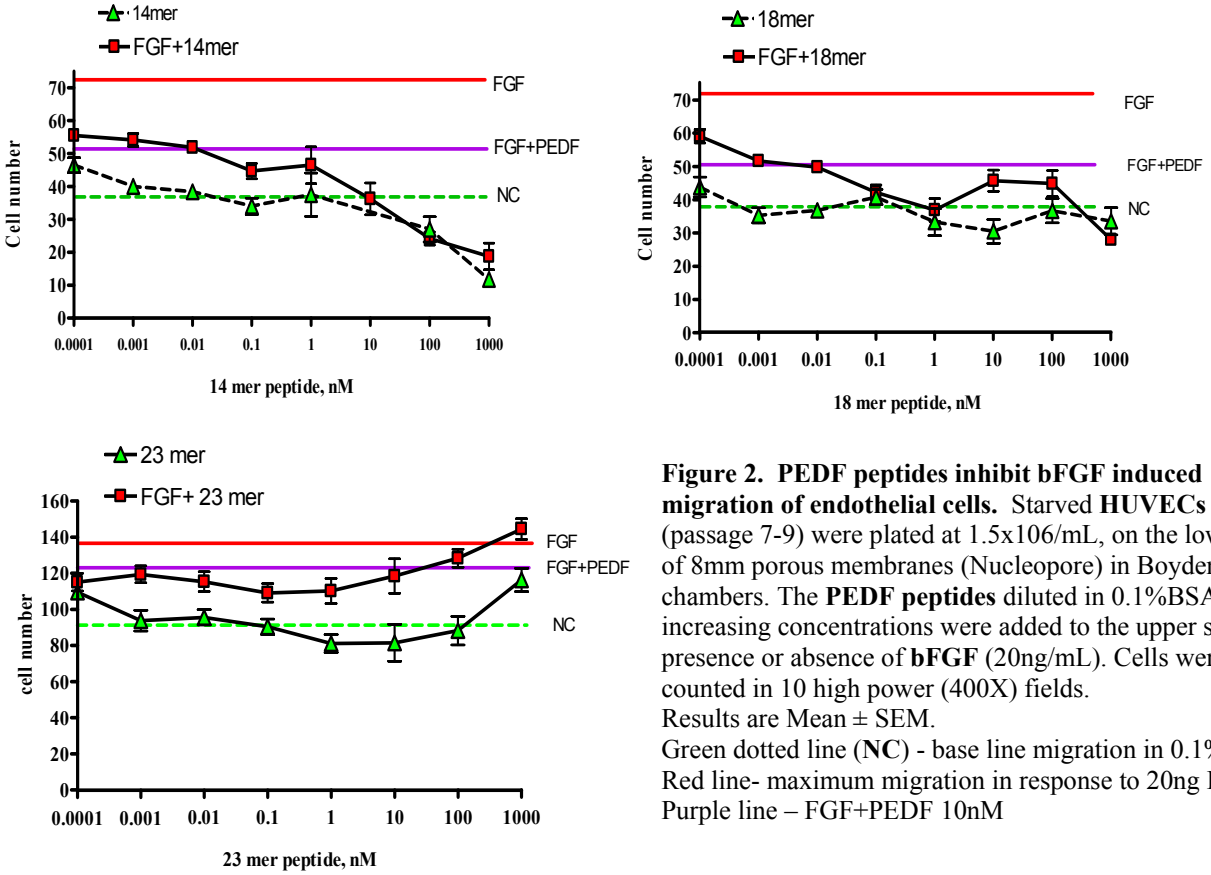
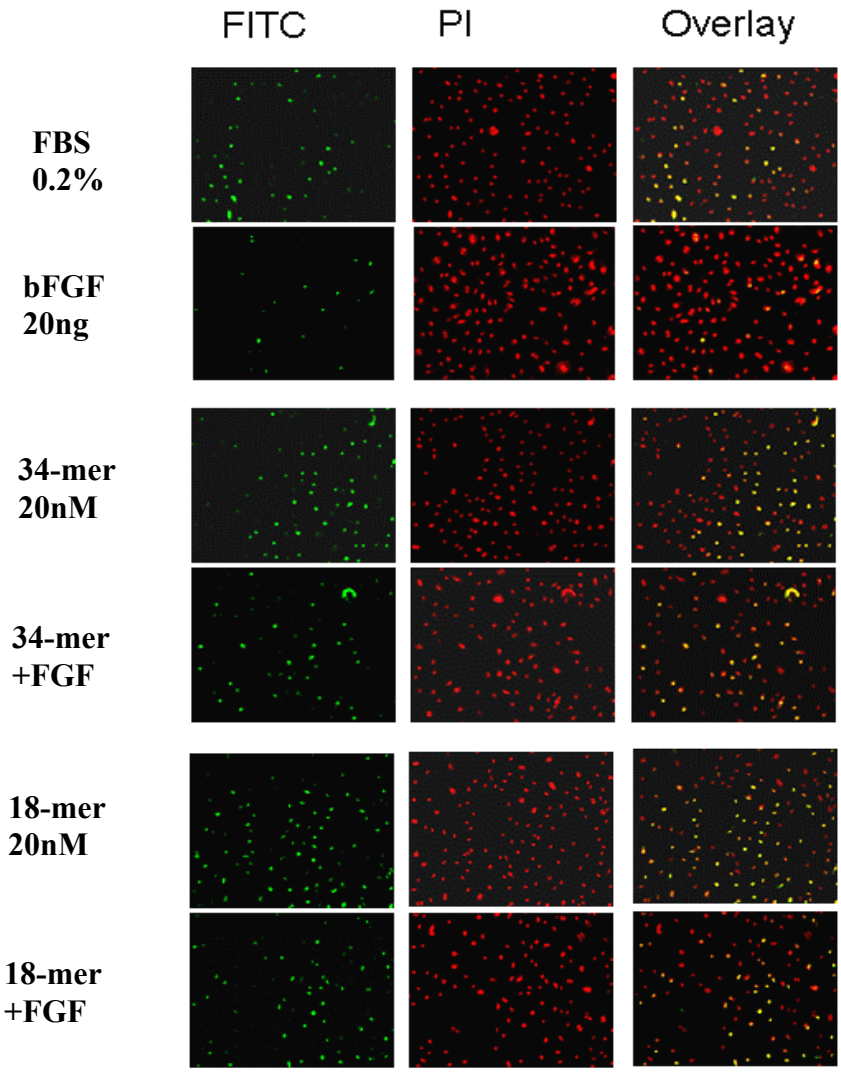


Figure 2. PEDF peptides inhibit bFGF induced migration of endothelial cells. Starved **HUVECs** (passage 7-9) were plated at 1.5x106/mL, on the lower side of 8mm porous membranes (Nucleopore) in Boyden chambers. The **PEDF peptides** diluted in 0.1%BSA at increasing concentrations were added to the upper side in presence or absence of **bFGF** (20ng/mL). Cells were counted in 10 high power (400X) fields.
Results are Mean ± SEM.
Green dotted line (**NC**) - base line migration in 0.1% BSA;
Red line- maximum migration in response to 20ng FGF;
Purple line – FGF+PEDF 10nM

All peptides inhibited bFGF induced migration of human human umbilical vein endothelial cells (HUVECs). Inhibitory effect was statistically significant according to One Way ANOVA ($p<0.05$) when compared to bFGF-induced migration (Fig.2). 14-mer and 18 mer were more effective than PEDF and demonstrated dose response in the concentration range between 0.1 nM and 10 nM. While 23-mer inhibited EC migration to the same extent as PEDF, its effect was not dose-dependent. When tested alone, 14-mer showed slight toxicity above 10nM, while 18 and 23 were not toxic at all the doses tested.

3. I determined the rate of EC apoptosis in response to the peptide treatment
As was previously demonstrated by our lab and by others (*1*) apoptosis of EC is the major mechanism by which PEDF blocks effect of angiogenic stimuli. I used terminal dUTP nick-labeling assay (TUNEL) to measure apoptosis. 34-mer has been previously shown to reproduce PEDF signaling events therefore in this study we evaluated the ability of the 34-mer derived peptides to elicit EC apoptosis (Fig. 3 A, B).

A


FITC
PI
Overlay
FBS
0.2%
bFGF
20ng
34-mer
20nM
34-mer
+FGF
18-mer
20nM
18-mer
+FGF

B

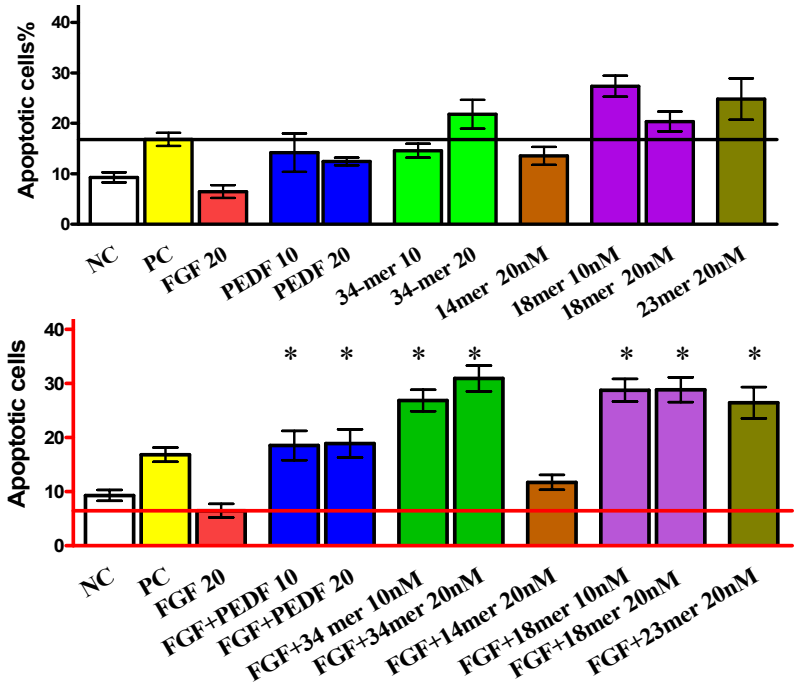
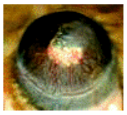
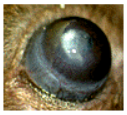
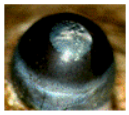
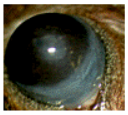
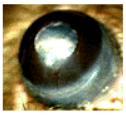

Apoptotic cells%
40
30
20
10
0
NC
PC
FGF 20
PEDF 10
PEDF 20
34-mer 10
34-mer 20
14mer 20nM
18mer 10nM
18mer 20nM
23mer 20nM
Apoptotic cells
*
NC
PC
FGF 20
FGF+PEDF 10
FGF+PEDF 20
FGF+34 mer 10nM
FGF+34mer 20nM
FGF+14mer 20nM
FGF+18mer 10nM
FGF+18mer 20nM
FGF+23mer 20nM

Figure 3. PEDF peptides block survival effect of bFGF by inducing endothelial cells apoptosis. A. Immunofluorescent staining of human endothelial cells. Confluent HMVEC cells growing on coverslips were incubated overnight in low serum media (0.2% FBS in MCDB) ± **PEDF peptides** in the presence or absence of **bFGF.** ApopTag **Fluorescein** kit (Chemicon) was used to determine apoptotic cells.
B. Quantitative analysis of apoptosis. 3-5 fields (10X) were analyzed with epi-fluorescent microscope, and cells were counted using MetaMorph software. Apoptotic (FITC positive) cells were calculated as a % of total cells stained with PI per field. Results are presented as Mean ± SEM. Bars marked with ∗ - statistically significant differences in comparison with FGF (by One Way Anova).

Overnight serum deprivaton (0.2% FBS) was used as a positive control (PC) for apoptosis. Adding FGF to the low serum media had protective effect: apoptosis was reduced to the level found in cells growing in full medium (10% FBS, negative control, NC). Cells treated with peptides alone (Fig 3B, upper panel) demonstrated levels of apoptosis similar to those in positive controls. When added in the presence of bFGF, 18- and 23-mer peptides significantly blocked its survival effect (verified by ANOVA). The pro-apoptotic activity of these peptides was comparable with 34-mer and PEDF. Interestingly, the shortest peptide, 14-mer, did not block b-FGF cell survival, although it was effective in migration assay.

4. I confirmed the ability of peptides to inhibit vascularization in mouse corneal assay. Based on the in vitro screening results we have chosen 18-mer peptide to be tested in the corneal assay and compare its effect to the 34-mer, which previously demonstrated activity comparable with PEDF (*1*). Peptides were incorporated in slow-release pellets, with or without bFGF. The pellets were implanted in mouse corneas and angiogenesis was scored on day 7 post implantation (positive corneas of total implanted, see Table 1 below). Two doses of the 18-mer were used in the corneal assay: both effectively blocked bFGF induced angiogenesis.

Table 1. PEDF peptides block FGF-induced angiogenesis

Peptides added	**FGF**	**FGF+ 34-mer 10μM**	**FGF+ 18-mer 1μM**	**FGF+ 18-mer 10μM**	**18-mer 10μM**
Representative pictures					
Number of corneas, Positive/Total	**6 / 7**	**2 / 11**	**1 / 10**	**0 / 7**	**0 / 8**
Fisher's exact test (p vs FGF)		**0.049**	**0.009**	**0.005**	**0.0014**

During the in vitro screening I encountered several problems some of which were anticipated. Human endothelial cells can undergo limited number of passages in culture (10-12) and their response to bFGF (or other stimuli) may vary depending on passage number. To reduce this variability we had to use cells within passages 6 to 9 for all experiments. In addition, because EC are pooled from different donors, the response varied from lot to lot and between suppliers. Another problem was the variability in the purity and activity of the commercially available PEDF, which we originally used as a control in all the experiments. Finally, I switched to the production and purification of recombinant PEDF from transfected HEK cells in our own lab. Although I have ultimately solved all these difficulties it increased the time allocated for the initial in vitro screening, compared to that projected in the initial SOW and the following steps 5 and 6 are not yet completed.

5. I will test chosen peptides in the quantitative matrigel angiogenesis assay (3 doses per compound). We projected 150 mice to be used (10 mice per dose). The plugs are excised at the endpoint. One-half of the plugs are used to quantify angiogenesis by hemoglobin content. The remaining plugs are snap-frozen, and retained for analysis.

6. Prepare and stain cryosections of the matrigel plugs for the endothelial cell marker, CD31, and detect apoptosis by TUNEL. Fluorescent images are obtained and quantified using LasePix software. The efficacy is determined as: (a) % decrease in hemoglobin content; (b) % decrease in MVD; (c) % increase in EC apoptosis.

In addition, new, more efficient "Directed in Vivo Angiogenesis Assay" (DIVAA^{TM}) is now offered by Trevigen Inc. It requires longer time to establish angiogenesis than plug assay (somewhere between 9 and 15 days), however it allows to reduce the number of animals per group; provides more quantitative and reproducible results (*2, 3, 4*), and greatly reduces time required for evaluation and analysis. Also, while C57/BL6J mice are common strain used in Matrigel plug assay, athymic nude mice present a better model with more consistent angiogenic response. We have performed a pilot experiment using DIVAA^{TM} and demonstrated the efficacy of 18mer, while the 23-merr failed to block angiogenesis induced by bFGF/VEGF combination. We would like to use this technique to in further experiments to quantify anti-angiogenic effect of the PEDF peptides.

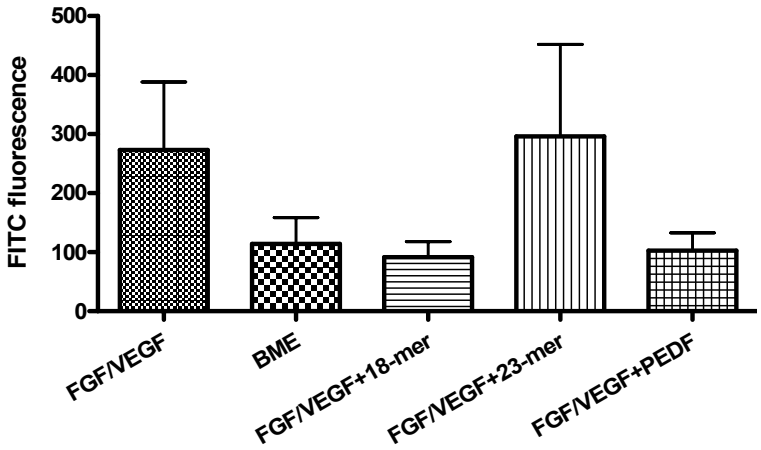


Figure 4 Evaluation of angiogenesis inhibition using DIVAA. DIVAA reactors containing basement membrane extract (BME), and/or pro-angiogenic factors (FGF/VEGF) in the presence or absence of PEDF peptides were implanted subcutaneously in nude mice (4 reactors/ mouse). In 12 days reactors were excised and processed as previously described (*2,4*). Results are Mean±SEM (n=8-11).
FGF/VEGF stimulated vascularization and addition of 18-mer peptide or PEDF blocked this effect reducing level of vascularization to the level of negative control (BME). 23-mer peptide did not block vascularization.

KEY RESEARCH ACCOMPLISHMENTS:

- ✓ In search of active anti-angiogenic epitope of PEDF I generated 3 short peptides covering the 34-mer anti-angiogenic fragment, which are potential candidates for anti-angiogenic therapies of prostate cancer.
- ✓ I performed initial screening of their in vitro activity and determined the most effective peptide (18-mer)
- ✓ I have demonstrated the ability of the 18-mer peptide to block neovascularization in vivo and compared it to the parental 34-mer peptide in corneal assay.
- ✓ I recommend this peptide for further testing in angiogenesis and tumorigenicity assays.

REPORTABLE OUTCOMES:

Abstract: Mirochnik Y, Filleur S, Volpert O. Pigment Epithelial derived factor: development of anti-angiogenic peptides. 2006. European J Cancer 4S (12):37

Presentation: Symposium on "Molecular Targets and Cancer Therapeutics". Prague, Czech Republic 7-10 November 2006

CONCLUSIONS: We designed and tested a number of peptides, which represent the active epitope of PEDF, a protein with anti-angiogenic, anti-tumor properties. One of the peptides is active at low nanomolar concentrations, with negligible toxic effects at higher concentrations and can therefore be developed as a novel anti-cancer agent. In addition, this active peptide may be used in co-crystallization studies with newly discovered PEDF receptor (*5*) in order to generate small molecule non-protein PEDF mimetics. Such small-molecule agents are more feasible from the economical and manufacturing standpoint.

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5. L. Notari *et al.*, *J Biol Chem* **281**, 38022 (Dec 8, 2006)

APPENDICES: See following pages

American Journal of Pathology, Vol. 162, No. 5, May 2003

Technical Advance

Quantitative Assessment of Angiogenic Responses by the Directed *in Vivo* Angiogenesis Assay

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From the Extracellular Matrix Section, Laboratory of Pathology, and the Vascular Biology Faculty,† Center for Cancer Research, National Cancer Institute; and the Division of Bioengineering and Physical Science,‡ Office of Research Services, National Institutes of Health, Bethesda, Maryland*

One of the major problems in angiogenesis research remains the lack of suitable methods for quantifying the angiogenic response *in vivo*. We describe the development and application of the directed *in vivo* angiogenesis assay (DIVAA) and demonstrated that it is reproducible and quantitative. This assay consists of subcutaneous implantation of semiclosed silicone cylinders (angioreactors) into nude mice. Angioreactors are filled with only 18 μl of extracellular matrix premixed with or without angiogenic factors. Vascularization within angioreactors is quantified by the intravenous injection of fluorescein isothiocyanate (FITC)-dextran before their recovery, followed by spectrofluorimetry. Angioreactors examined by immunofluorescence show cells and invading angiogenic vessels at different developmental stages. The minimally detectable angiogenic response requires 9 days after implantation and ≥50 ng/ml ($P < 0.01$) of either fibroblast growth factor-2 or vascular endothelial growth factor. Characterization of this assay system demonstrates that the FITC-labeled dextran quantitation is highly reproducible and that levels of FITC-dextran are not significantly influenced by vascular permeability. DIVAA allows accurate dose-response analysis and identification of effective doses of angiogenesis-modulating factors *in vivo*. TNP-470 potently inhibits angiogenesis (EC_{50} = 88 pmol/L) induced by 500 ng/ml of fibroblast growth factor-2. This inhibition correlates with decreased endothelial cell invasion. DIVAA efficiently detects differences in anti-angiogenic potencies of thrombospondin-1 peptides (25 μmol/L) and demonstrates a partial inhibition of angiogenesis (~40%) in a matrix metalloprotease (MMP)-2-deficient mouse compared with that in wild-type animals. Zymography of angioreactors from MMP-deficient and control animals reveals quantitative changes in MMP expression. These results support DIVAA as an assay to compare potencies of angiogenic factors or inhibitors, and for profiling molecular markers of angiogenesis *in vivo*. (Am J Pathol 2003, 162:1431–1439)

Therapeutic targeting of angiogenesis is a novel approach to cancer therapy that has achieved proof-of-principle in animal models of tumor progression.[1–4] The recent discovery of specific molecular targets that modulate endothelial cell responses has given further impetus for the development and therapeutic application of angiogenesis-targeted therapy.[3,4] However, one of the major problems in angiogenesis research, both at the basic and applied levels, remains the development of suitable methods for assessing and quantifying the angiogenic response *in vivo*.[5,6]

In the adult population angiogenesis is the principal mechanism leading to the formation of a functional and well-developed blood supply.[7,8] Angiogenesis refers to the formation of endothelial sprouts from existing capillary networks. Targeting blood vessel development associated with chronic diseases in the adult, such as ischemic cardiovascular disease, cancer, and arthritis, has focused on therapeutic intervention in the process of vascular sprout formation.[4,8]

Several key steps in the process of endothelial sprouting have been identified.[2,8] The process begins with vasodilatation and an increase in vascular permeability, initiated by vascular endothelial growth factor (VEGF) produced in response to hypoxia. Increased vascular permeability results in extravasation of plasma proteins that become organized into a provisional matrix that, along with the subendothelial basement membrane, form a physical barrier that must be crossed by migrating

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endothelial cells. Changes in the adhesion of endothelial cells to the extracellular matrix, induction of protease activities and cell migration, as well as endothelial cell proliferation have also been reported. Finally, endothelial sprouts undergo lumen formation, anastomosis, and initiation of blood flow.

Investigators have developed both *in vitro* and *in vivo* assays that allow further characterization of the cellular events described above.[5,6] However, *in vitro* angiogenesis assays, based on endothelial cell cultures or tissue explants, focus on isolated endothelial cell functions (eg, endothelial cell proliferation, migration, or invasion) and do not examine the coordination of cell functions required for a successful angiogenic response.[5,6,9,10] In contrast, *in vivo* angiogenesis assays examine the entire spectrum of molecular and cellular processes, with the endpoint being formation of new, functional blood vessels. However, these *in vivo* angiogenesis assays are not only expensive and technically difficult to perform, but also require substantial amounts of test compound and most rely on selective morphometric analysis (eg, vessel counts, vascular morphology, and so forth) for quantitation.[5,6] Because of these limitations, current drug development strategies for identification and testing of novel angiogenic compounds and inhibitors depend principally on the use of *in vitro* systems. Although *in vitro* angiogenesis assays have been useful for identification of potential molecular targets to augment, alter, or block endothelial cell responses and preliminary screening of novel pharmacological agents, they frequently cannot be correlated with *in vivo* angiogenesis measurements.[9,10] This is most likely the result of the complex and multiple cellular mechanisms evoked during new blood vessel formation *in vivo*.

The current approaches to development of targeted therapeutic agents for angiogenesis, and further understanding of pathogenic mechanism of angioproliferative diseases lack an *in vivo* assay that utilizes technically simple, reproducible methodology that is easily quantified and independent of morphological parameters.[6,11] Such an *in vivo* assay would complement the current *in vitro* approach to development of novel angiogenic agents, as well as to study the temporal and spatial expression *in vivo* of molecular markers in pathological angiogenesis. We describe the development and application of a novel, murine *in vivo* angiogenesis assay that is reproducible, and quantitative that requires only microliter assay volumes. We demonstrate the utility of this assay in characterization of an induced angiogenic response to known angiogenic factors, as well as inhibition of this response by several compounds with different mechanisms of action.

Materials and Methods

Reagents

Matrigel and fibroblast growth factor (FGF)-2 were obtained from Collaborative Research (Becton Dickinson, Bedford, MA). Recombinant, murine VEGF-A was obtained from R&D Systems (Minneapolis, MN). TNP-470 was obtained from the Developmental Therapeutics Program, Frederick Cancer Research and Development Center (FCRDC), National Cancer Institute, Frederick, MD. Purified, human platelet thrombospondin-1 (TSP-1), as well as D-reverse peptides p416 and p545, derived from the central stalk region of TSP were kindly supplied by Dr. David Roberts, Laboratory of Pathology, National Cancer Institute. Adrenomedulin was obtained from Peninsula Laboratories (San Carlos, CA), fluorescein isothiocyanate (FITC)-labeled dextran was obtained from Sigma Chemical Co. (St. Louis, MO) and FITC-labeled *Griffonia (Bandeiraea) simplicifolia* lectin I, isolectin B4 (FITC-lectin) was obtained from Vector Laboratories (Burlingame, CA).

Directed in Vivo *Angiogenesis Angioreactor*

Sterile, surgical silicone tubing (0.15-cm internal diameter) was cut to standard 1-cm lengths using a Plexiglas template and single edge razor blade. These were plugged at one end with a 1/16 inch outer diameter × 4-mm solid stainless steel rod or seal with silicone, and sterilized by steam autoclave. These are referred to as "angioreactors." Using a Hamilton syringe, sterilized angioreactors were filled at 4°C with 18 μl of Matrigel with or without angiogenic factors. These were incubated at 37°C for 1 hour to allow gel formation, before subcutaneous implantation into the dorsal flank of C57/BL6, C57/BL6 MMP-2-deficient (provided by Dr. David Muir, University of Florida, Gainesville, FL) or athymic nude mice (females, 6 to 8 weeks of age; National Cancer Institute, Frederick, MD). Before collection of the angioreactors, mice received a 100-μl injection of 25 mg/ml of FITC-dextran in phosphate-buffered saline (PBS) via tail vein. Quantification was performed by removal of the Matrigel and digestion in 200 μl of Dispase solution (Collaborative Research) for 1 hour at 37°C. After digestion, the incubation mix was cleared by centrifugation (5 minutes at 15,000 × *g*, room temperature in a benchtop centrifuge (Eppendorf/Brinkman. Westbury, NY) and fluorescence of the supernatant aliquots were measured in 96-well plates using an HP model spectrofluorimeter (excitation 485 nm, emission 510 nm; Perkin Elmer, Boston, MA). The mean relative fluorescence ± SD for five replicate assays were determined. Statistical analysis (nonparametric Student's *t*-test; nonlinear regression, one-site competition) were performed using Prism Software Package for Macintosh (Graph Pad, San Diego, CA).

Characterization of Vascular Permeability during DIVAA

The contributions of vascular permeability to the FITC-dextran signal during quantification of angiogenic responses in the DIVAA assay were determined. The time course of FITC-dextran accumulation within the angioreactor in response to 500 ng/ml of either FGF-2 or VEGF were obtained at 9 days after implantation in angioreactors containing either FGF-2 or VEGF. Mice were injected

intravenously with 100 μl of FITC-labeled dextran by tail vein. Angioreactors were then recovered at 10, 30, and 45 minutes and 1 hour after intravenous injection. FITC-dextran levels were assayed after Dispase digestion by fluorescence spectrometry as described.

Endothelial Cell Invasion Assay

FITC-labeled *Griffonia* lectin (FITC-lectin), an endothelial cell selective reagent, was used to quantify invading endothelial cells into the Matrigel. Briefly, after recovery of DIVAA angioreactors and digestion with Dispase as described above, cell pellets and insoluble fractions were collected by centrifugation and 5000 × *g* for 2 minutes at room temperature in a tabletop centrifuge. The cell pellets were resuspended in 1 ml of phosphate-buffered saline (PBS) and washed three times with PBS. After the final wash the cells were again collected by centrifugation and resuspended in 200 μl of 25 μg/ml of FITC-lectin and incubated at 4°C overnight. The stained cell pellets were again centrifuged and washed three times with cold PBS. The final pellet was resuspended in 100 μl and relative fluorescence was determined for triplicate assays as described above. Mean relative fluorescence units ± SD were determined as above.

Histological Examination

Nine days after implantation, angioreactors together with the immediate surrounding tissue were dissected and fixed in 10% neutral buffered formalin. Histological sections of paraffin–embedded assays were prepared by 10-μm sectioning and stained by conventional hematoxylin and eosin methods. Sections were also stained using *Griffonia* lectin (FITC-lectin). Stained sections were examined and photographed using a Zeiss Axioscope fluorescent microscope with a digital camera attachment (Spot model 1.3.0; Diagnostic Instruments, Sterling Heights, MI). The FITC-dextran signals within whole implants were examined using an inverted fluorescent microscope (Olympus IX70) and photographed as above.

Gelatinase Activity

Biochemical analysis of the gelatinase (MMP-2 and MMP-9) activity was performed by zymogram analysis.[12] Matrigel is removed from recovered implants and resuspended in 200 μl of PBS. After mechanical disruption with a pipette tip samples were centrifuged (15,000 × *g* for 5 minutes at room temperature, tabletop centrifuge). Aliquots of the supernatant were prepared with 2× Novex Tris-glycine sample buffer (Invitrogen, Carlsbad, CA) and applied to Novex 10% zymogram gels. Electrophoresis and zymogram analysis were performed as previously described.[12]

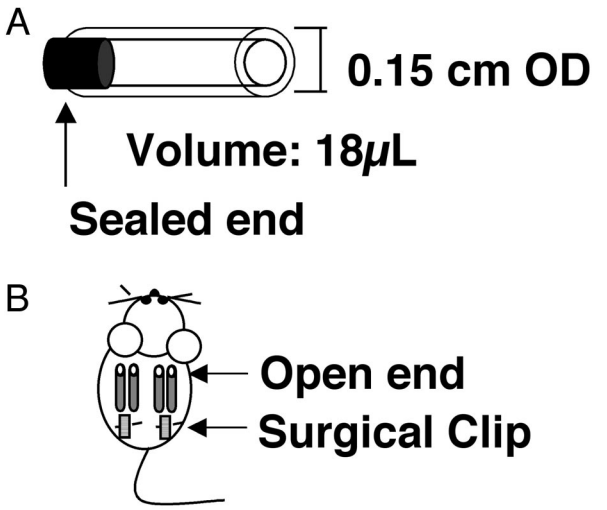

Figure 1. DIVAA. Angioreactor: A 0.15 cm × 1-cm long silicone rubber tubing is plugged at one end and filled with 16 μl of Matrigel with or without FGF-2 or VEGF as explained in Materials and Methods (**A**). Multiple angioreactors are implanted subcutaneously into the dorsal areas of athymic nude or C57/BL6 mice. Typically two assays are implanted per mouse with their open ends away from the surgical incision as depicted, but as many as four (two per dorsal flank) have been implanted per animal (**B**).

Results

Directed in Vivo *Angiogenesis Assay (DIVAA) Design*

Our objective in the development of the DIVAA is to replicate the angiogenic response induced during chronic disease states. In these pathological conditions angiogenesis develops in response to an imbalance of positive and negative effectors that results in a gradient of positive angiogenic stimulus, with the direction of initial endothelial sprouting occurring along the axis of this gradient.[2] A relevant *in vivo* model should reproduce (or amplify) this vectorial component of the angiogenic response. Several geometric configurations (discoid, spheroid, and so forth) were tested before selecting a cylindrical shape generated by a section of silicone tubing. This configuration amplified the directional response to angiogenic stimuli. Subsequent modifications minimize the assay volume and amount of test material required. The final prototype configuration of the angioreactor utilizes a 1-cm segment of sterile silicone tubing sealed at one end containing a specified volume (18 μl) of reconstituted extracellular matrix premixed to give a final fixed concentration of angiogenic factor(s) (Figure 1). The present report exclusively utilizes Matrigel, although alternative extracellular matrix gels (eg, fibrin or type I collagen) are also functional in this assay (data not shown). All data generated in the present report, with the exception of those obtained in the MMP-2 knockout experiments, are obtained using a single, Matrigel-containing angioreactor per mouse. Up to four assays per mouse, two on each side of the dorsal midline, can be implanted without an adverse effect on the interassay variation (data not shown).

Kinetics of Vascularization

At various times after implantation (2 to 15 days), angioreactors were dissected and visually inspected for angiogenesis. Vascularization within the angioreactor was readily observed at day 6 after implantation. Figure 2 shows both FGF-2 and control (without exogenous angiogenic factor) assays from various time points after subcutaneous implantation in athymic nude mice. With increasing duration of implantation, the angiogenic response within the FGF-2-containing angioreactors (upper row) progressively invades deeper into the Matrigel. Measurement of the linear extent of angiogenesis within the angioreactor (Figure 2, bottom) demonstrates that a stable difference between the FGF-2 assay and negative control is achieved at approximately day 9. Nine days after implantation, the occasional presence of an angiogenic response is observed in a few negative control assays. However, the extent of this response is usually minimal and clearly much later in the time course than the response obtained with FGF-2-positive controls. Matrigel is an extract of basement membrane proteins from the murine Englebreth-Holm-Swarm tumor.[13] There are reports of minimal angiogenic responses to Matrigel alone, because of the presence of low concentrations of endogenous growth factors, either co-purified or bound to ex-

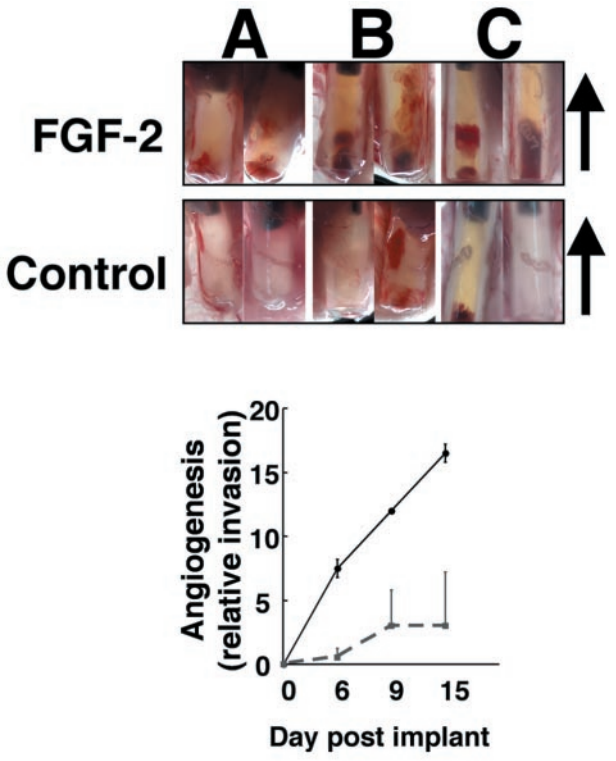

Figure 2. Kinetics of angiogenesis invasion into FGF-2-containing DIVAA. Angioreactors were prepared and implanted as described. On removal from the mouse, angioreactors are photographed using a Leica MZ125 microscope connected to a digital camera (Spot; Diagnostic Instruments, Sterling Heights, MI). **Top**: Paired angioreactors recovered at 6 (**A**), 9 (**B**), and 15 days (**C**) after implantation with FGF-2 or without (control) containing Matrigel. Angioreactors are oriented with open end at bottom and sealed end at top, **arrow** indicates direction of invasion from open end. Progression of angiogenic response is observed with increased vascular tissue within the lumen. **Bottom**: The relative distance of the extent of invasion of the vascular response from the open end of the assay is plotted *versus* the day of recovery after implantation. A mild angiogenic response is observed in two of four DIVAAs recovered at days 9 and 15 after implantation into athymic nude mice. We selected day 9 for further characterization of the assay. Original magnifications, ×10.

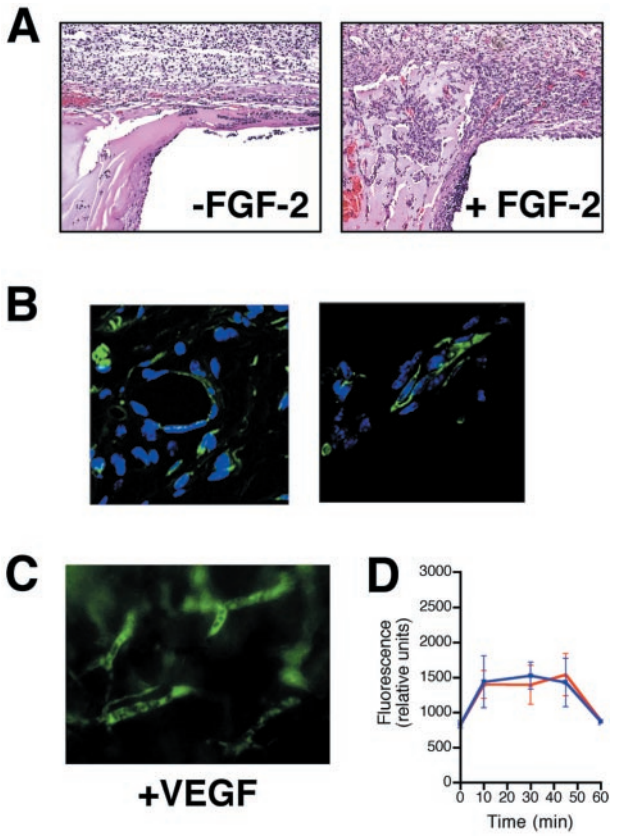

Figure 3. Histology and immunofluorescence microscopy of angioreactors. **A:** H&E-stained section from control (−FGF-2) and angiogenic factor-containing (+FGF-2) implants (day 9) showing interface at open end. Loose granulation tissue containing a modest mononuclear cell infiltrate is observed. However, there is only occasional cellular invasion of the Matrigel, with no evidence of vascular invasion. In the section from the FGF-2 (500 ng/ml) implant the granulation tissue response at the open end of the implant is enhanced and numerous vascular structures, some containing red blood cells are obvious, both within the granulation tissue as well as invading the Matrigel. Overall the invading vascular structures are oriented parallel to the long axis. **B:** Section from Matrigel of FGF-2-containing angioreactor stained for endothelial cells with *Griffonia* lectin (FITC-lectin). Cell surface of endothelial cells are positive for FITC-lectin (green signal). Sections are counterstained with DAPI for nuclear localization (blue signal). The figures show FITC-lectin-positive organizes vascular structures with lumen formation, as well as clusters and individual endothelial cells that are not yet organized into vascular structures. **C:** Fluorescence microscopy of FITC-dextran signal. Angioreactors containing either FGF-2 or VEGF were examined as whole mount preparations without fixation using an inverted fluorescent microscope. Representative images of angioreactor containing VEGF (500 ng/ml) (+VEGF) showing clearly demarcated vascular structures present only in the VEGF containing angioreactor, confirming the quantitation of fluorescent signal as representative of vascular volume within the angioreactor. **D:** Time course analysis of FITC-dextran response in FGF-2- and VEGF-induced angiogenesis. Angioreactors containing either FGF-2 or VEGF were prepared and implanted as described. At 9 days after implantation, mice were injected intravenously with FITC-labeled dextran. At indicated time points after intravenous FITC-dextran injection, angioreactors were recovered and analyzed for FITC-dextran by fluorescence spectrometry. The levels of FITC-dextran remain steady during the 10 to 45 minutes time course of this experiment. This includes the 20-minutes time point after FITC-dextran used for routine analysis in other experiments. At the 1-hour time point levels of FITC-dextran are decreased to background levels, demonstrating that no significant levels of FITC-dextran accumulate within the angioreactors. Blue, FGF-2-induced angiogenesis; Red, VEGF-induced angiogenesis. Original magnification, ×200 (**C**).

tracellular matrix components in the Matrigel preparations.[5]

Histological Examination

Angioreactors recovered at 9 days reveal a mild inflammatory infiltrate (granulation tissue) in both the FGF-2 and control (without angiogenic factor) assays (Figure 3A). However, unlike the histology of control angioreactors with no angiogenic factor, assays containing an angiogenic factor (FGF-2) reveal numerous blood vessels near the open end (compare Figure 3A), that invade the Matrigel to form vascular structures at various stages of development. Vascular structures containing red blood cells within the FGF-2/Matrigel angioreactors are readily appreciated (Figure 3A). In addition, the response to FGF-2-containing angioreactors also shows clear organization in the direction of the long axis, consistent with the directed nature of the response. Matrigel invasion and early organization of endothelial cell-containing structures is revealed by immunofluorescent staining with fluorescein *Griffonia (Bandeiraea) simplicifolia* lectin 1 staining (isolectin B4), an endothelial cell selective marker.[14,15] This staining reveals endothelial cell invasion into angioreactors containing angiogenic factor, both as individual and multicellular aggregates, with and without lumen formation (Figure 3B).

Quantification of Angiogenesis

Direct quantification of angiogenic response is obtained by intravenous injection of FITC-dextran (average molecular weight, 150,000 d) 20 minutes before sacrificing the mice, as explained in Materials and Methods. Quantitation of the fluorescence within angioreactors reflects the internal vascular volume and is expected to correlate with the extent of the angiogenic response provided that there is no significant leakage of the FITC-dextran. Examination of the FITC-dextran fluorescence in whole mount samples of angioreactors using either FGF-2 or VEGF as an angiogenic stimulus revealed sharply delineated vascular structures without leakage of FITC-dextran into the angioreactor's perivascular space, Figure 3C.

To specifically examine the possible contribution of vascular permeability and/or leakage of FITC-dextran to the angiogenic responses, we determined the time course of FITC-dextran that accumulates within the angioreactor after intravenous injection. The results of this experiment are shown in Figure 3D. The data clearly demonstrate that between 10 and 45 minutes, which includes the 20-minute time point used for routine analysis, the levels of FITC-dextran within the angioreactor remain constant in response to either FGF-2- or VEGF-induced angiogenesis. However, by 60 minutes, the levels of FITC-dextran have decreased in both the FGF-2- and VEGF-induced assays to baseline levels (present in angioreactors without angiogenic factors). This observation suggests that the FITC-dextran is cleared from the functional vessels within the angioreactor and that the level of FITC-dextran leakage is undetectable over background. The results of this experiment conclusively demonstrate that the angiogenic response determined by intravenous FITC-dextran quantification is not significantly altered by changes in vascular permeability and/or the use of FITC-labeled dextran (molecular weight, 150,000). This result does not preclude the presence of vascular permeability but does demonstrate that these effects are not of sufficient magnitude to be detectable in the present assay system. These findings probably account for the highly consistent and reproducible responses observed in the DIVAA system.

Furthermore, there is a direct correlation between the extent of the angiogenic response within the implant and the amount of angiogenic factor used in the assay system. The results of dose-response experiments using both FGF-2 and VEGF are shown in Figure 4A. The results demonstrate that DIVAA requires doses higher than 50 ng/ml for both angiogenic factors. However, at a dose of 500 ng/ml of FGF-2 is slightly more potent than the equivalent concentration of VEGF in ng/ml. Although, on a molar basis VEGF would be approximately three times more potent in this assay system.

DIVAA Interassay Variation

We characterize the interassay variation using FITC-dextran. Figure 4B shows FITC-dextran quantification of angiogenesis at 9 day after implantation for six independent experiments (comparing quintuplicate FGF-2 *versus* quintuplicate negative controls in each experiment). These data demonstrate consistently high levels of fluorescence in angioreactors containing FGF-2 as compared with the negative control assays (without FGF-2) and that the difference between the means for the FGF-2-containing and negative controls readily achieve statistical significance in five of six experiments ($P < 0.01$; only experiment 2 fails). In addition, the collective individual data points from all six experiments are pooled and tested in a two-way analysis of variance (variation around the mean and between assays). The low interassay variation (two-way analysis CV = 10%, 95% confidence interval = 8.3 to 13%) demonstrates that the assay described above is a useful system for comparison of relative effectiveness of different angiogenesis agents or inhibitors performed in independent experiments. We subsequently refer to this assay, using our designed angioreactors and FITC-dextran for quantitation, as the directed, *in vivo* angiogenesis assay, or DIVAA.

Endothelial Cell Responses in DIVAA

Intravenous injection of FITC-dextran is a measurement of blood volume within the angioreactor and is not necessarily a direct measure of endothelial cell responses. To directly demonstrate the endothelial cell responses, and correlate this with the DIVAA determinations, we use fluorescein-labeled *Griffonia (Bandeiraea) simplicifolia* lectin 1 (FITC-lectin) to quantify murine endothelial cell numbers within the angioreactor.[14,15] The results of the FITC-lectin determination of endothelial cell invasion are

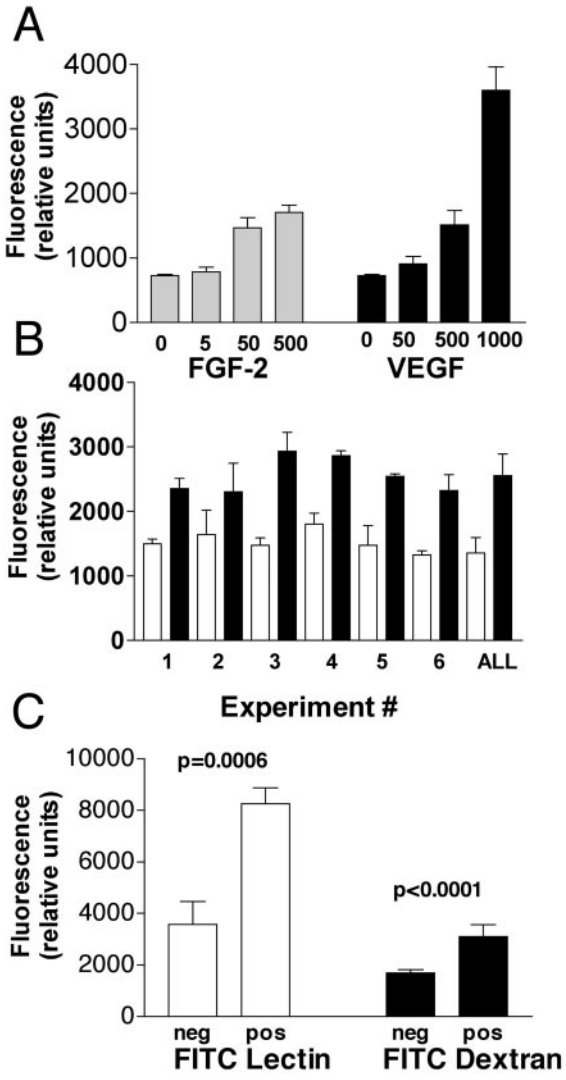

Figure 4. Quantification of DIVAA by FITC-dextran injection and validation of endothelial responses. Angioreactors are prepared and surgically implanted subcutaneously in nude mice as in Materials and Methods. **A:** Before recovery of the angioreactors each animal received a single tail-vein injection of 100 μl of 25 mg/ml FITC-dextran dissolved in phosphate-buffered saline. Twenty minutes after this injection the animals were sacrificed and the angioreactors recovered. Responses in both FGF-2 (5 to 500 ng/ml)- and VEGF (50 to 1000 ng/ml)-containing Matrigel and Matrigel without angiogenic factors at 9 day after implantation are determined by fluorescence quantitation of FITC-dextran after Dispase digestion, as explained in Materials and Methods. Results are expressed as relative fluorescence units of FITC-dextran. The results demonstrate that the level of angiogenic response is proportional to the dose of angiogenic factor in both FGF-2- and VEGF-containing angioreactors. **B:** Results of six independent experiments, each using five replicate determinations, comparing response to negative control (**white bars**) with FGF-2-induced angiogenesis (**black bars**). Student's *t*-test comparison of results for FGF-2 and negative controls indicates statistically significant differences ($P < 0.01$) in five of six experiments (only experiment 2 failed). In addition pooled results from all six experiments were tested for variation around the mean and interassay variation. The two-way coefficient of variation (CV) for the pooled assays is ~10% (95% confidence interval = 8.3 to 13%). This low CV value indicates that DIVAA is suitable for interassay comparison of results. **C:** Comparison of FITC-lectin (endothelial cell response) and DIVAA in control assays. Angioreactors with (pos) and without (neg) FGF-2 are prepared and implanted for 9 days as before. After recovery of angioreactors, the angiogenic response is quantified by DIVAA or for direct endothelial cell invasion using FITC-lectin staining of Dispase cell pellets, as described in Materials and Methods. Results demonstrate that the FGF-2 (positive) angioreactors contain increased numbers of FITC-lectin-stained endothelial cells and FITC-dextran signal than implants without this well-characterized angiogenic factor. Although the signal intensity is greater in the endothelial invasion assay (FITC-lectin), there are also larger SDs (error bars) and background (greater signal in negative control) associated with this assay.

compared with results from an identical DIVAA (Figure 4C). The assay of endothelial cell invasion using the FITC-lectin again demonstrates statistically significant differences between positive (FGF-2-containing) and negative controls with slightly higher variation (larger SD, error bars) compared with DIVAA (FITC-dextran). These data demonstrate that DIVAA, using FITC-dextran for quantitation, is representative of a true endothelial cell response, as shown by FITC-lectin staining of invasive endothelial cells, which results in new vessel formation as observed in histological sections (Figure 3).

Accurate Determination of the TNP-470 Anti-Angiogenic Activity by DIVAA

Fumagillin, isolated from the *Aspergillus fumigatus*, is known to be a potent anti-angiogenic compound.[16] In 1992, TNP-470, a synthetic analogue of fumagillin, entered clinical trials as the first anti-angiogenic agent.[17,18] Basic and clinical research efforts support TNP-470 as a prototype anti-angiogenic agent.[19] The efficacy of TNP-470 to inhibit angiogenesis is examined in DIVAA and the FITC-lectin assay of endothelial cell invasion, Figure 5. Angioreactors containing FGF-2 with or without varying concentrations of TNP-470 are prepared as described above. Results using DIVAA indicate that TNP-470 inhibits the FGF-2-induced angiogenic response with an $EC_{50} = 88 \times 10^{-12}$ mol/L. This value compares favorably with EC_{50} ~600×10^{-12} mol/L in the FITC-lectin assay and the low pmol/L IC_{50} reported for *in vitro* inhibition of endothelial cell growth.[16] Comparison of the FITC-lectin measurement of endothelial cell invasion with the DIVAA (FITC-dextran) determinations demonstrates that the FITC-lectin assay is somewhat less sensitive to TNP-470 inhibition. This decreased sensitivity of the FITC-lectin assay is probably because of the requirement for a threshold number of endothelial cells to be present within the angioreactor before detection of a fluorescence signal over the background. However, these results confirm our conclusion that DIVAA represents a true angiogenic response involving an increase in the number of invading endothelial cells, and that these invasive endothelial cells are seen isolated or forming vascular structures of varying stages of development (Figure 3).

Comparison of DIVAA with Other in Vivo *Angiogenesis Assays*

To demonstrate that DIVAA can be used to quantify the angiogenic response involving other mechanisms of angiogenesis inhibition, we examined the activity of peptide fragments of an endogenous angiogenesis inhibitor, as well as a genetic model involving inactivation of the matrix metalloproteinase-2 (MMP-2) expression.

TSP-1 is an extracellular matrix glycoprotein that functions to modulate cell-matrix interactions[20] and has been shown to inhibit endothelial cell function *in vitro*, as well as angiogenesis using both *in vivo* and *ex vivo* techniques.[20–24] The anti-angiogenic activity of TSP-1 has

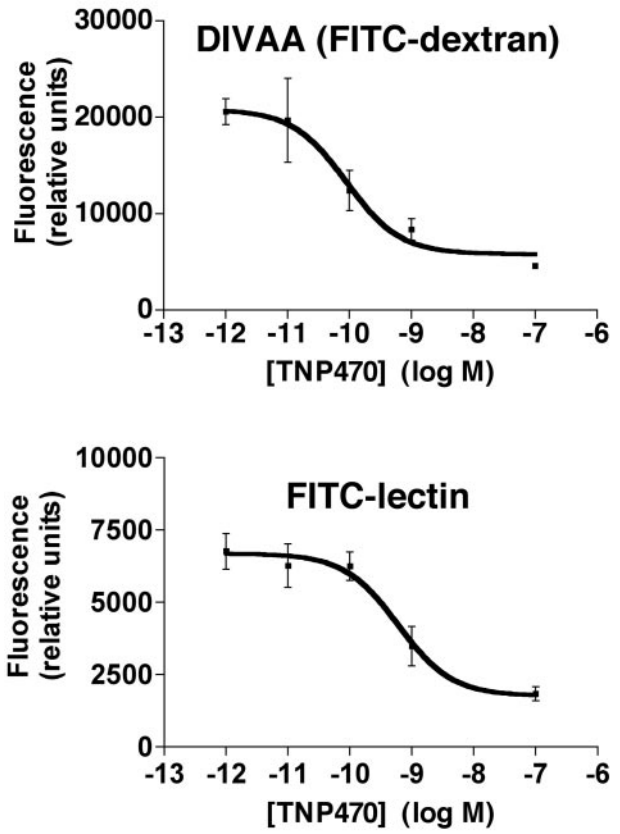

Figure 5. DIVAA determination of EC_{50} for TNP-470. Angioreactors containing FGF-2 with indicated concentrations of TNP-470 were prepared and assayed at 9 days as before. Results show a dose-response of decrease in the angiogenic response with increasing TNP-470 concentration. DIVAA determination reveals an EC_{50} = 88 pmol/L with a high correlation for the curve fit analysis (R^2 = 0.91). Complete inhibition of the angiogenic response in this assay is observed around 10 nmol/L. These results compare favorably with those obtained using the FITC-lectin determination of endothelial cell invasion (**bottom**). In the FITC-lectin assay the TNP-470 EC_{50} = 600 pmol/L, also with a high coefficient curve fitting (R^2 = 0.94), but maximal inhibition was observed at ~100 nmol/L concentration of TNP-470.

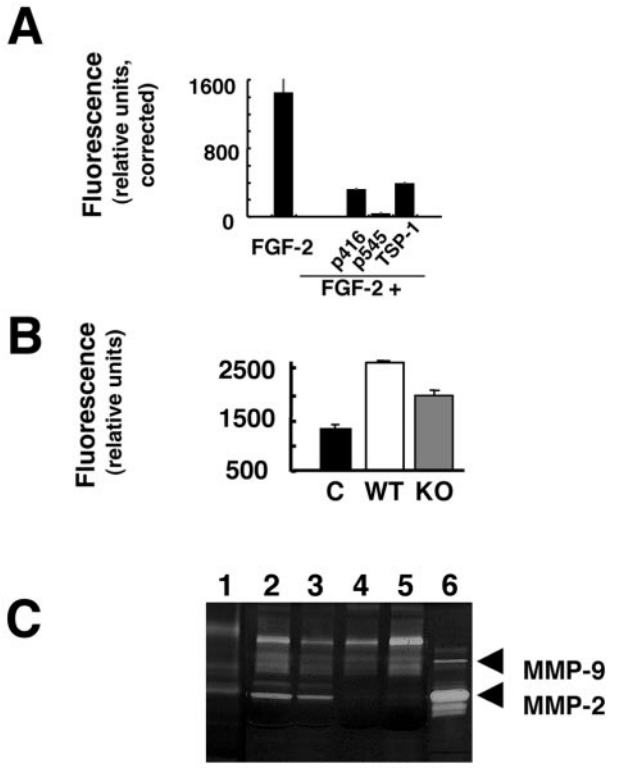

Figure 6. DIVAA characterization of angiogenesis inhibition. To demonstrate that DIVAA is useful in a characterization of a variety of angiogenesis inhibitors experiments using endogenous angiogenesis inhibitors (TSP-1) and a genetic model (MMP-2-deficient murine model) are conducted. **A**: Angioreactors are prepared with FGF-2 alone (FGF-2), FGF-2 plus 0.1 μmol/L final concentration of TSP-1 (FGF+TSP-1), as well as FGF-2 plus 25 μmol/L final concentration of the following TSP-1 peptides, p 416, p545, and p641. DIVAA is performed as described. Results are corrected for negative control (Matrigel without FGF-2) and plotted as relative fluorescence units. DIVAA results demonstrate ~60% inhibition by 0.1 μmol/L TSP-1, with the following rank order of TSP-1 peptides (25 μmol/L final concentration) for inhibition of angiogenesis: p545> p416. These results are consistent with previous reports describing the relative anti-angiogenic potency of TSP-1 peptides. Data represent the mean ± SD of five replicate determinations. **B**: DIVAA quantitation of angiogenesis in a murine gene-targeted model. C57/BL6 mice with targeted disruption of the matrix metalloproteinase-2 (MMP-2) gene (KO) and wt C57/BL6 (WT) mice were implanted with angioreactors containing FGF-2 or without (negative control, **C**) and the angiogenic responses quantitated by DIVAA. No detectable differences in the negative controls (WT *versus* KO) are observed. The FGF-2-induced angiogenic response in KO mice is reduced by 35% compared with responses observed in WT mice ($P < 0.01$), consistent with previous reports. Data represent mean ± SD of five replicate determinations. **C**: Comparison of MMP-2 and MMP-9 expression in angioreactors recovered from WT and KO mice by zymogram analysis.[12] **Lane 1**, negative control, Matrigel alone; **lanes 2** and **3**, angioreactors from WT mice; **lanes 4** and **5**, implants from KO animals; **lane 6**, MMP-2 and MMP-9 standards. Results show complete loss of MMP-2 (pro- and active forms) in KO mice without significant change in MMP-9 expression. Zymogram gel loading is normalized to total protein concentration.

been assigned to specific domains within the molecule.[20,22] The anti-angiogenic effects of native soluble TSP-1 and two TSP-1 peptides (25 μmol/L final concentration) are tested using DIVAA (Figure 6A). TSP-1 at a 0.1 μmol/L final concentrations significantly inhibits the angiogenic response to FGF-2 (~60%) in experiments using DIVAA. Potent inhibition of FGF-2 induced angiogenesis is also observed with TSP-1 peptides p545 (~97% inhibition) and p416 (~78% inhibition). These results validate the anti-angiogenic effects of TSP-1 peptides reported using other *in vivo* angiogenesis assays and demonstrate the relative efficacy of different TSP-1 domains to inhibit FGF-2-induced angiogenesis. Moreover, previous reports using the neonatal rat retinal angiogenesis[21] and chick chorioallantoic membrane[22] assays to demonstrate the anti-angiogenic activity of TSP-1 peptides required 10-fold more peptide for these assays than DIVAA. These results suggest that anti-angiogenic agents of diverse mechanisms of action can be characterized using DIVAA. Most importantly, quantification of the angiogenic response by DIVAA is objective and does not require multiple observers (as in the retinal angiogenesis assays), the use of digital image analysis as in the chick chorioallantoic membrane assay,[21,22] or selection of areas for vessel counts. DIVAA is an observer-independent and objective assay that requires substantially less test material. All these are critical parameters for an *in vivo* quantitative assay of angiogenesis.

Angiogenesis involves remodeling and invasion of the extracellular matrix. Like tumor cell invasion, matrix metalloprotease (MMP) activities, particularly MMP-2, MMP-9, and MT-1-MMP, are pivotal during angiogenesis.[25–27] We use DIVAA to quantify angiogenesis in mice

with a homozygous MMP-2 gene deletion and compare this response with that of wild-type animals (Figure 6B). FGF-2-induced vascularization is decreased in the MMP-2 knockout mice ($P < 0.01$) to 35% of the response observed in wild-type animals (Figure 6B). This is in agreement with a previous study in which MMP-2-deficient animals demonstrated a 31% reduction in angiogenic response (quantitated by vessel counting) compared to wild-type animals.[27] The results of these studies confirm that MMP-2 plays at least a partial role in the angiogenesis induced by FGF-2.

The DIVAA technique also allows material contained in the assay to be recovered for additional biochemical, cellular, or genetic testing. To demonstrate this, DIVAA from both MMP-2 mutant and wild-type mice are recovered and analyzed by zymogram for MMP-2 and MMP-9 activity. As shown in Figure 6C, expression of pro- or active MMP-2 is completely absent in the knockout animals. Levels of pro- or active MMP-9 inside the angioreactor did not change in the MMP-2 knockout mice in comparison to levels present in wild-type control experiments. These findings are consistent with previous reports in which MMP-2 and MMP-9 are shown to function independently of one another during tumor-induced angiogenesis.[28] This analysis of the DIVAA demonstrates the potential for detailed molecular analysis of cellular and tissue mechanisms involved during the angiogenic response. Thus DIVAA provides an opportunity to examine, at the molecular level, multiple new targets and/or markers for anti-angiogenic therapy in cancer patients.

Discussion

Despite important breakthroughs in angiogenesis research, animal models that allow objective and quantitative assessment of the *in vivo* angiogenic response remains as the major limitation to the development of effective anti-angiogenic therapies.[5,6,9,11] Currently, novel angiogenesis targeted therapies lack *in vivo* screening models suitable for objective, quantitative preclinical testing, making it difficult to obtain dose-response analyses and estimate therapeutic doses before initiating clinical trials.[4–6] Previous *in vivo* assays of angiogenesis, although helpful in developing our understanding of the angiogenic process, have proven difficult to objectively quantify.[5,6,11]

This report describes development of the DIVAA that meets the criteria for quantification that is essential for a useful preclinical model of angiogenesis. The geometry and semienclosed configuration of the angioreactor maintains constant and constrained matrix volume, fixed concentration of angiogenic factor(s), as well as defined local concentration of test compounds. In addition, the angioreactor's design makes it possible to amplify the angiogenic or anti-angiogenic effects in a small test volume ($<20\ \mu l$), as well as to organize and direct the growth of angiogenic vessels. All this is what differentiates DIVAA from previously described *in vivo* angiogenesis assays. In summary, our findings demonstrate that DIVAA: 1) recapitulates the vectorial component (directional response) that is characteristic of angiogenesis associated with chronic disease states; 2) is objectively quantifiable and highly reproducible; 3) directly correlates with increased numbers of endothelial cells within the extracellular matrix of the assay and that these invading endothelial cells form vascular structures of varying degrees of maturity (solid endothelial cell aggregates, isolated single cell invasion, and mature vascular structures with lumen formation and red blood cells); 4) is highly sensitive to effects of angiogenesis inhibitors; thus, it is useful to generate dose-response analysis in preclinical testing of angiogenesis inhibitors; and 5) can be used with angiogenesis inhibitors of diverse mechanisms of action or genetic backgrounds (MMP-2-deficient mouse) to quantitatively measure and compare angiogenic responses. The correlation between the responses observed in the DIVAA with the direct assessment of endothelial cell invasion (FITC-lectin assay) demonstrates that DIVAA quantitation represent a true endothelial cell-mediated angiogenic response. Furthermore, we demonstrate that recovery of the DIVAA angioreactors can facilitate further biochemical analysis (protease activity, and so forth) of the angiogenic response. Ongoing experiments further demonstrate that we can monitor the effectiveness of systemic administration of angiogenesis inhibitors using DIVAA as well as analysis of angioreactors by microtechniques for gene expression profiling (cDNA and protein microarrays). Progress in development of angiogenesis-targeted therapies is hampered by the lack of appropriate *in vivo* assays. The DIVAA provides a reproducible model that is objectively quantifiable and in which the directed response simulates the spectrum of endothelial cell responses associated with vascular sprouting. The design of this assay should prove a useful *in vivo* research tool for mechanistic studies of pro- and anti-angiogenic agents, and molecular profiling of angiogenesis, as well as a potential screening assay for drug development.

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